PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/00, 38/18, 31/70 — A1

(11) International Publication Number: **WO 97/17983**

(43) International Publication Date: 22 May 1997 (22.05.97)

(21) International Application Number: PCT/US96/18031

(22) International Filing Date: 12 November 1996 (12.11.96)

(30) Priority Data:
60/006,541 13 November 1995 (13.11.95) US

(71) Applicants *(for all designated States except US)*: CAMBRIDGE NEUROSCIENCE, INC. [US/US]; Building 700, One Kendall Square, Cambridge, MA 02139 (US). UNIVERSITY OF VIRGINIA PATENT FOUNDATION [US/US]; Suite 1-110, 1224 West Main Street, Charlottesville, VA 22903 (US). E.K. SHRIVER CENTER FOR MENTAL RETARDATION [US/US]; 200 Trapelo Road, Waltham, MA 02254 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MARCHIONNI, Mark, A. [US/US]; 24 Twin Circle Drive, Arlington, MA 02174 (US). MAHANTHAPPA, Nagesh, K. [US/US]; 240 Norfolk Street, Cambridge, MA 02139 (US) SCHWARTING, Gerald [US/US]; 49 Old Orchard Lane, Boxborough, MA 01719 (US). CORWIN, Jeffrey [US/US]; 278 Turkey Ridge Road, Charlottesville, VA 22903 (US).

(74) Agent: BUTLER, Gregory, B., Cambridge NeuroScience, Inc., Building 700, One Kendall Square, Cambridge, MA 02139 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report

(54) Title: METHODS OF TREATING DISORDERS OF NON-VISUAL SENSORY EPITHELIA

(57) Abstract

In general, the present invention provides methods for promoting the function of inner ear cells using neuregulins. A novel aspect of the invention involves the use of neuregulins as growth factors to promote function of non-visual sensory epithelial cells. Treating of the non-visual sensory epithelial cells to provide these effects may be achieved by contacting non-visual sensory epithelial cells with a polypeptide described herein. The treatments may be provided to slow or halt net cell loss or to increase the amount or quality of non-visual sensory epithelial tissue present in the vertebrate.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia
AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

METHODS OF TREATING DISORDERS OF NON-VISUAL SENSORY EPITHELIA

GOVERNMENT SUPPORT

This invention was made with the support of federal grants from the U.S. Government. The Government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates to methods of affecting function of non-visual sensory epithelial cells, including, but not limited to, cells of the vestibular, cochlear and olfactory epithelia.

BACKGROUND OF THE INVENTION

The invention relates to prophylactic or affirmative treatment of diseases and disorders of non-visual epithelia and associated tissues of the sensory organs by administering polypeptides found in vertebrate species, which polypeptides are growth, differentiation and survival factors for several cell types. Normal function of sensory epithelia cells including survival, proliferation, differentiation, and maintenance is dependent upon the controlled expression of a variety of peptide growth factors. Some of these factors can be produced by neuronal cells and by other cells of the sensory epithelia,which provide a signal to regulate sensory epithelial cell function.

I. Anatomy and function of sensory epithelia

A. Hearing and the Sense of Balance

The senses of hearing and balance depend upon the normal development and activity of tissues that comprise the auditory and vestibular pathways, respectively. The auditory system consists of the outer ear (the external ear and tympanic membrane), the middle ear (location of the middle ear bones: malleus, incus, and stapes) and the cochlea of the inner ear (see Figure 1). The sensory cells that respond to sound, the hair cells, reside within the cochlea and transform sound information into electrical impulses that are transmitted to the brain via

give rise to the sense of balance and head position. These hair cells are similar to cochlear hair cells, and transmit electrical signals to the brain via neurons of the vestibular ganglion. These two systems share a common embryological origin, and are physiologically similar

1. Anatomy and function of the cochlear-vestibular epithelia

The Cochlear Epithelium

The cochlea of mammals is a spiral structure containing two fluid-filled tubes, the scala vestibuli and the scala tympani (see Figure 2).

These parallel tubes wrap around a central bony pillar (the modiolus) two and a half times, and connect with each other at the apex (via the helicotrema) of the cornucopia-shaped complex. When sound impinges upon the eardrum it sets the bones of the middle ear vibrating, and they in turn vibrate the membranous oval window. Since the scala vestibuli begins at the oval window, fluid within the tubes is set into motion and the scalae vestibuli and tympani vibrate in unison. The vibrations dissipate at the terminus of the scala tympani, the membranous round window.

A third tube, the cochlear duct, is closed at both ends and is sandwiched between the above-described tubes. The floor of the cochlear duct is called the basilar membrane, and on this membrane sits the organ of Corti. The organ of Corti constitutes the primary sensory receptor surface of the auditory system; one row of inner hair cells and three to four rows of outer hair cells run the entire length of this epithelium. The hair cells are surrounded by support cells known as pillar cells, Deiter's cells, Henson's cells and phalangeal cells, and these are attached to the cartilaginous basilar membrane.

The hair cells are mechanosensitive cells that bear cilia ("hairs") on their apical surfaces. These cilia contact an overlying membrane such that vibrations moving through the scalae cause the cilia to move back and forth. The back and forth motion causes the opening and closing of apical ion channels, and thus cochlear hair cells respond to sound by electrical depolarization and hyperpolarization. At their basal surfaces, hair cells form chemical synapses with neural fibers originating from the spiral ganglion, and these changes in polarization are communicated to the central nervous system by release of a neurotransmitter.

The three tubular structures within the cochlea are narrowest towards the oval and round windows, and broaden towards the apex. This structure confers a critical mechanical property on the system; when sound sets the fluid within the scalae vestibuli and tympani in motion, a

[illegible] reduces sounds maximally so[illegible] the narrower portion of the organ of Corti. The various regions of the organ of Corti are preferentially stimulated by

specific frequencies of sound, thus in humans a continuous "tonotopic" field (ranging in responsiveness from 20 Hz to 20,000 Hz) is mapped over the length of the epithelium. Perceived loudness is proportional to the amplitude of the sonic vibrations. In summary, perception of tone is a function of which hair cells are stimulated, and perception of loudness is a function of how strongly the given hair cells are stimulated.

The vestibular system works in a similar fashion, but rather than making use of a topographic continuum to encode information, vestibular hair cells are segregated into five distinct substructures that respond to distinct stimuli. The hair cells of the vestibular system are embedded in gelatinous material such that those within the vestibule respond to linear/translational motion, and those within the semicircular canals respond to rotational/angular motion. The extracellular gel encasing hair cells within the vestibule contain calcium carbonate crystals (otoliths) that directly stimulate the hair cells by falling on them. As with cochlear hair cells, the hair cells of the vestibular system form synapses with fibers from the vestibular ganglion to transmit information to the central nervous system.

Cochlear Innervation and the Primary Auditory Pathway

The circuitry of the auditory pathway, the path by which hair cell responses are transmitted through the brain, is quite complex. An overview of this circuitry follows. Neurons of the spiral ganglion are bipolar, extending one process to synapse with hair cells and another process that splits and synapses with two cochlear nuclei (ventral and dorsal) within the ipsilateral brainstem. Hair cell signals therefore pass through two synapses to reach neurons within the brain. The projection from cochlea to cochlear nuclei is known as the VIIIth cranial nerve. (It is worth noting that an efferent, inhibitory pathway synapses primarily onto outer hair cells, and that these axons are of olivary origin. These are thought to play a feedback role in modulation of hair cell responsiveness.)

Neurons of the cochlear nuclei project contralaterally to the inferior colliculus (IC), a region responsible for auditory reflexes such as the startle response. Some axons projecting to the IC send branches to other brain nuclei including the contralateral nucleus of the lateral lemniscus (NLL). Some neurons of the NLL project to the ipsilateral IC, while the ipsilateral and contralateral IC communicate directly through the commissure of the IC. Thus at this level of the auditory system, connections cross heavily and information from both ears is available to both sides of the brain. IC neurons send non-crossing projections to the medial

extraction., etc.)

Two properties of auditory projections are of particular interest. First, auditory projections throughout the nervous system maintain tonotopic alignment. In other words, if one were to measure electrical responses to various pure tones in nuclei of the primary auditory pathway, one would find response properties within individual nuclei distributed along an axis corresponding to the axis of tonotopy found in the organ of Corti. Second, the "tuning curves" of single auditory neurons sharpen as one ascends the auditory pathway. This is to say that auditory hair cells respond to a increasingly broad range of frequencies as sound amplitude increases, but higher order auditory neurons exhibit increasingly specific frequency responses. At the cortical level, auditory neurons display specificities for increasingly complex temporal patterns of frequency modulation.

The VIIIth cranial nerve also contains axons originating from the vestibular system. As with the auditory system, the neurons of the vestibular ganglion send one process to the hair cells, and one process that projects to the cerebellum and four vestibular nuclei in the ipsilateral brainstem. The vestibular nuclei project broadly throughout the brain stem (where vestibular information is integrated with oculomotor information, etc.) and into the spinal cord (where vestibular information contributes to the modulation of posture and muscle tone).

B. The senses of smell and taste

Both the sense of smell and a large part of what is considered the sense of taste are mediated by the olfactory system. In the case of smell, airborne volatile molecules (odorants) are inhaled through the nose and sensed by a discreet patch of sensory epithelium (the olfactory epithelium) that resides in the rearmost region of the nasal passages upon bony ridges known as turbinates. Though the fundamental flavors identified as sweet, sour, salty and bitter are detected by sensory cells of the tongue, the bulk of gustatory experience is the product of flavor molecules volatilizing in the oral cavity and interacting with the olfactory epithelium via passages connecting the oral and nasal cavities. Thus, a normal sense of smell and taste is dependent upon the normal development and function of the olfactory epithelium and its connections to the central nervous system.

1. Anatomy of the olfactory system

The architecture of the [illegible]

[illegible] Figure 1 shows a schematic diagram of the olfactory epithelium. The deepest layer, that which abuts the bony turbinates, is referred to as the lamina propria

and contains primarily connective and vascular tissues. Separating the lamina propria from the actual sensory epithelium, or neuroepithelium, is the basal lamina. The basal lamina appears to be a typical, acellular, basement membrane comprised of many extracellular matrix proteins such as collagens, laminins, and fibronectin.

The neuroepithelium is populated by multiple cell types that are restricted in their laminar position. Immediately adjacent to the basal lamina are the basal cells; those abutting the basal lamina are known as horizontal basal cells, and those sitting somewhat less tightly upon the horizontal basal cells are known as globose basal cells. Between the basal cell layer and the most superficial layer of the olfactory epithelium lie the cell bodies of the primary olfactory neurons. The olfactory neurons are the actual chemosensory cells that detect odorants and transmit odor information to the central nervous system. This function depends on two extensions from each neuron cell body: a single dendrite that extends towards the nasal cavity where it is capped by cilia that bear odorant receptor proteins (see below); and a single axon that extends in the opposite direction through the basal cells and basal lamina and via the lamina propria connects directly to neurons of the olfactory bulb in the central nervous system. The binding of specific odorants by specific odorant receptors causes the neuron to depolarize and thus signal the olfactory bulb though an action potential that travels the olfactory axon. "Support" cells of unknown function, called sustentacular cells, fill in around the olfactory dendrites and constitute the bulk of the exposed epithelial surface. In addition there are the cells that make up Bowman's glands - mucus secreting glands that are periodically distributed across the breadth of the olfactory epithelium and that span the depth of the neuroepithelium.

III. Peptide Growth Factors

The development and physiology of multicellular organisms requires multiple modes of intercellular communication. Such communication may be systemic, as in the case of hormones delivered via the bloodstream, or can be highly localized. In the latter case, two modes are commonly recognized: synaptic signaling from neurons; and paracrine signaling from adjacent or nearby cells (Alberts et al., *Molecular Biology of the Cell*, 2nd ed. Garland Publishing, New York, NY, 1989). A function of such signaling is to coordinate cell survival, proliferation, differentiation, and/or metabolic activity. The molecules that serve as transmitted signals vary in their chemical composition; one group of molecules are proteins, the peptide growth factors. Peptide growth factors act upon cells by binding to cell surface receptors. These receptors are coupled to intracellular signal transduction pathways that give

A. Peptide Growth Factors in the cochlear-vestibular epithelia

Three independent lines of evidence provide strong support for the role of the neurotrophic factors NT3 and BDNF in the development of the inner ear. The studies include: *in situ* hybridization to map the factor NT3 and BDNF (Pirvola, U., Ylikowki, J., Palgi, J., Lethonen, E. Arumae, U. and Saarma, M., *PNAS USA* 89: 9915-9919, 1992) and the receptors trkB and trkC (Enfors, P., Merlio, J.-P., and Persson, H. *Eur. J. Neurosci.* 4:1140-1158, 1992); culture studies demonstrating that BDNF and NT3 support survival and neurite extension of developing cochlear vestibular ganglia neurons (Avila, M. A., Varela-Nieto, I., Romero, G., Mato, J. M., Giraldez, F., Van De Water, T. R. and Represa, J. *Dev. Biol.* 159:266-275, 1993), and; analysis of mice deficient in BDNF (Ernfors, P., Lee, K.-F., Kucera, J. and Jaenisch, R. *Cell* 77:503-512, 1994; Jones, K. R., Farinas, I., Backus, C. and Reichardt, L. F. *Cell* 76:989-999, 1994) and NT3 (Ernfors, P., Lee, K. F. and Jaenisch, R. *Nature* 368:147-150, 1994; Farinas, I., Jones, K. R., Backus, C., Wang, X. Y. and Reichardt, L. F. *Nature* 369:658-661, 1995) showed deficits in innervation and hair cell morphology.

Principal sites of NT3 expression include both support cells and hair cells of developing cochlear sensory epithelium and basal cells of the vestibular epithelium. The expression of BDNF is localized to sensory cells (hair cells), but is not found in the support cells of the sensory epithelium. Thus, the distributions of these two neurotrophins are overlapping, but not identical. Neurons of the spinal ganglia and the vestibular ganglia, which innervate the sensory epithelia of the cochlear and the three epithelium of the vestibular system, respectively, have been shown to express trkB and trkC receptors (Enfors, P., Merlio, J.-P., and Persson, H. *Eur. J. Neurosci.* 4:1140-1158, 1992).

In concert with the known distribution of neurotrophins and trk receptors, the results from gene knockout experiments have led to the conclusion that BDNF is the major survival factor for vestibular ganglia neurons and NT3 for spinal ganglion neurons (Ernfors, P., Lee, K.-F., Kucera, J. and Jaenisch, R. *Cell* 77:503-512, 1994; Ernfors, P., Lee, K. F. and Jaenisch, R. 1994; Enfors, P., Van De Water, T., Loring, J., and Jaenishce, *R. Neuron* 14:1153-1164, 1995).

A variety of culture systems have been used to study the activities of neurotrophins on cells of the inner ear and their [illegible]

[illegible] Lethonen E. Arumae [illegible] and Saarma, M. *PNAS USA* 89 9915-

9919, 1992; Avila, M. A., Varela-Nieto, I., Romero, G., Mato, J. M., Giraldez, F., Van De Water, T. R. and Represa, J. *Dev. Biol.* 159:266-275, 1993).

More recently, BDNF, NT3 and NT4/5 have been shown to promote survival of cultured vestibular ganglion neurons and to protect them from cell death induced by cytotoxins (Zheng, J. L., Stewart, R. R. and Gao, W.-Q. *J. Neurobiology* 28:3, 330-3401, 1995).

Specific growth factors that stimulate hair cell regeneration have not been identified.

B. Peptide Growth Factors in the olfactory system

In a serum-free culture system, Mahanthappa and Schwarting demonstrated that epidermal growth factor (EGF) promotes basal cell division and transforming growth factor βs (TGF-βs) promote neurogenesis (Mahanthappa, et al. *Neuron* 10:293-305, 1993). Whether EGF plays the role of basal cell mitogen *in vivo*, is not known. Furthermore, it remains to be determined whether EGF or another member of the EGF superfamily of peptide growth factors (Massague, et al. *Ann Rev Biochem* 62:515-541, 1993) is actually responsible for basal cell proliferation. Within the embryonic mouse olfactory epithelium, the most differentiated zones show high levels of TGF-β2 signal by in situ hybridization (Millan, et al. *Develop* 111:131-144, 1991). Thus, the finding that TGF-β2 promotes neurogenesis in the cultured olfactory epithelium and is more potent than TGF-β1 is entirely consistent with known patterns of localization *in vivo* (Mahanthappa, et al. *Neuron* 10:293-305, 1993). Furthermore, the finding that antibody-neutralization of TGF-β2 completely blocks baseline levels of olfactory neurogenesis *in vitro* supports the hypothesis that TGF-β2 is in fact the endogeneously produced neurogenic factor in the OE (Mahanthappa, et al. *Neuron* 10:293-305, 1993).

Though neurotrophins are expressed in the olfactory bulb (Guthrie, et al. *J Comp Neurol* 313:95-102, 1991), a potential source of trophic support, neither nerve growth factor nor brain-derived growth factor promote survival of olfactory neurons *in vitro* beyond one week (Mahanthappa, et al. *Neuron* 10:293-305, 1993). In a culture system established by Pixley (Pixley *Neuron* 8:1191-1204, 1992), cultured epithelium demonstrated a tremendous degree of neurogenesis when cultured upon a monolayer of astrocytes. Furthermore, co-culture with astrocytes induced the expression of olfactory marker protein (OMP), a maturation marker. Though Pixley has shown that astrocyte-conditioned medium is [illegible] and survival [illegible] identified

IV. Pharmaceutical need for treating disorders of the ear

A. Hearing Loss

More than twenty-eight million Americans experience hearing loss. Two million lack sufficient hearing to understand speech and three-hundred and fifty thousand are unable to hear anything. Twenty percent of Americans with hearing problems are of school age.

There are two major forms of hearing loss. Conductive hearing loss may be brought about by blockade of the ear canal, lesions of the ear drum, or lesions of the middle ear bones. Much progress has been made in treating these forms of hearing loss primarily through surgical intervention and the creation of biocompatible prostheses. Such forms of hearing loss, however, constitute the minority.

Eighty percent of patients with significant hearing loss, approximately seventeen million Americans, suffer from sensorineural hearing loss. This loss of auditory sensitivity is due to damage to hair cells and/or lesions of neurons in the primary auditory pathway. The primary cause of sensorineural hearing loss is hair cell death. Hair cell death can have a variety of causes: loud sound, aminoglycoside antibiotic treatment, and aging among them. Hair cell loss has long been considered permanent in mammals, thus in addition to sophisticated electronic hearing aids, an increasingly pursued therapeutic approach to sensorineural hearing loss is the use of cochlear implants. Such implants are placed within the cochlea and serve to directly stimulate the VIIIth nerve in response to sound. The degree to which such devices succeed in improving patient quality of life is highly variable. Recent studies, however, suggest that hair cell loss may not be permanent and thus may herald therapies that promote full functional recovery through promotion of hair cell regeneration.

A summary of these and other relevant statistics follow:

- More than 28 million Americans have a hearing loss; 80 percent of those affected have irreversible and permanent hearing damage.
- More than 1/3 of the U.S. population has a significant hearing impairment by age 65.
- Approximately 2 million people [illegible]
- [illegible] infants is born totally deaf.

- At least 1 million children are deaf or have a communication disorder.
- One of every 22 infants has hearing problems.
- Genetic factors are known to cause over 50 percent of all cases of severe childhood deafness.
- The average age of diagnosis of hearing loss is close to age 3.
- Sensorineural damage (damage to the hair cells and cochlea caused by genetics or exposure to noise) is the largest, single form of hearing loss affecting 17 million Americans.
- At least 15 percent of the U.S. population is affected by tinnitus.
- Persons over age fifty are twice as likely to have tinnitus.
- Otitis media accounted for 10 million visits to doctors' offices in 1975.
- Presbycusis affects 1/3 of the U.S. population over age 65.
- Meniere's syndrome causes bilateral hearing loss in 5 to 20 percent of cases.
- The estimated cost of care per year for persons with hearing impairment is $56 billion (based on $2,000 per patient annual costs for special education, speech therapy, hearing aids, physician and specialist fees, and other expenses).

B. Balance disorders

Over 90 million Americans, age 17 and older, have experienced a dizziness or balance problem. In the United States, there are an estimated 97,000 cases of Meniere's disease each year.

The above information was obtained from the National Strategic Research Plan published by the NIDCD, NIH, April 1989

C. Therapeutic need for olfaction

The sense of smell is a primary mediator of appetite. Patients undergoing chemotherapy for cancer treatment are often treated with high doses of anti-mitotic agents, and among the many side effects of such agents is the loss of appetite. This loss of appetite is most often attributed to the severe disruption of the gastrointestinal tract since these tissues undergo continual mitotic renewal, and anti-mitotic agents interfere with their normal functioning. Keeping in mind that the primary olfactory neurons of the olfactory epithelium also undergo mitotic renewal, it is reasonable to expect that anti-mitotic chemotherapeutic agents would interfere with both the senses of taste and smell.

SUMMARY OF THE INVENTION

In general, the present invention provides methods for promoting the function of non-visual epithelial cells using neuregulins. A novel aspect of the invention involves the use of neuregulins as growth factors to promote function of inner ear cells. Treating of the inner ear cells to provide these effects may be achieved by contacting inner ear cells with a polypeptide described herein. The invention teaches how to stimulate mitogenesis of inner ear cells using neuregulins and therefore, treatments may be provided to slow or halt net cell loss or to increase the amount or quality of inner ear tissue present in a vertebrate. The invention also teaches how to promote survival of olfactory neurons using neuregulins and therefore, treatments may be provided to slow or halt net cell loss or to increase the amount or quality of olfactory tissue present in the vertebrate.

Neuregulins are a family of protein factors heretofore described as glial growth factors, acetylcholine receptor inducing activity (ARIA), heregulins, neu differentiation factor, which are encoded by one gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding to and activation of erbB2 (neu) and closely related receptors erbB3 and erbB4. The invention provides methods for using all of the known products of the neuregulin gene, as well as other not yet discovered splicing variants of the neuregulin gene. Thus, the above factors, regulatory compounds that induce synthesis of these factors, and small molecules which mimic the effect of these factors by binding to the receptors on non-visual sensory epithelial tissues or by stimulating through other means the second messenger systems activated by the ligand-receptor complex are all extremely useful as prophylactic and affirmative therapies for

The survival of non-visual epithelial cells as used herein refers to the prevention of loss of non-visual sensory epithelial cells by necrosis or apoptosis or the prevention of other mechanisms of cell loss. Survival, as used herein, indicates a decrease in the rate of cell death of at least 10%, more preferably by at least 50%, and most preferably by at least 100% relative to an untreated control. The rate of survival may be measured by counting cells stainable with a dye specific for dead cells (such as propidium iodide) in culture.

Methods for treatment of diseases or disorders using the polypeptides or other compounds described herein are also part of the invention. Examples of inner ear tissue disorders that may be treated, include inner ear diseases and disorders resulting from sensorineural pathologies, such as loss of hearing and/or balance, which may also be treated using the methods of the invention. These disorders of the inner ear include, but are not necessarily limited to: sensorineural hearing loss caused by ototoxic antibiotics, excessive noise, viral infection or autoimmune disease; tinnitus; and, Meniere's syndrome. Thus, administration of neuregulin in a therapeutically effective amount can provide a treatment for disorders of the ear, which otherwise left untreated would result in the loss of hearing and/or balance.

The methods of the invention make use of the fact that the various neuregulin proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding to $p185^{erbB2}$ (or related receptors erbB3 and erbB4) and activation of the same. Products of this gene are used to show inner ear cell mitogenic activity (see Examples 1 and 2, below). This invention provides a use for all of the known products of the neuregulin gene (described herein and in the references listed above), which have the stated activities as promoting inner ear cell function. Most preferably, recombinant human GGF2 (rhGGF2) is used in these methods.

The invention also relates to the use of other, not yet naturally isolated, splicing variants of the neuregulin gene. The splicing variants are fully described in Goodearl et al., US Patent No. 5,530,109, issued June 25, 1996 (formerly USSN 08/036,555, filed March 24, 1993), incorporated herein by reference.

Currently, there are limited options for therapy for the promotion of inner ear cell function, including survival, proliferation, differentiation, growth and changes in gene activity and metabolic activity. Such a therapy would be useful for treatment of a variety of inner ear

vertebrate

Advantages of the present invention include the development of new therapeutic approaches to injury or diseases of the ear, more specifically degenerative diseases of the cochlear-vestibular epithelia, based on the promotion of inner ear cell function through the use of neuregulins. Loss of inner ear cells is a common feature of degenerative ear diseases, and there are no available treatments, including growth factors. The factor can be formulated for injection and administered to patients that suffer from degenerative disorders, which lead to loss of hearing and/or balance. Thus, this approach to therapy can halt or slow the progressive loss of hearing and/or balance, which ensues in various ear diseases.

By analogy to the diseases of the ear, additional advantages of the invention include treatments for any type of non-visual sensory epithelia, such as diseases of olfactory, gustatory and other non-visual sensory epithelia. Agents that can promote olfactory neuron survival are likely to restore, at least in part, normal chemosensation and thereby facilitate recovery of normal appetite. This restoration of normal appetite is nontrivial, as the inability of patients undergoing chemotherapy to maintain reasonable levels of nutrition often complicates recovery. As neuregulins in the form of rhGGF2 can promote olfactory neuron survival, it may be an important adjunct therapy for such patients. Anosmia, the lack of a sense of smell, is also known to be a source of depression in patients who are otherwise normal. Again, for the reason that it promotes olfactory neuron survival, neuregulins may prove as useful adjunct therapeutics in the context of rhinal and/or psychiatric dysfunction.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagram of the gross anatomy of the ear.

Figure 2 is a diagram of the cochlea in cross-sectional view that depicts the organ of Corti, which contains the sensory hair cells.

Figure 3 is a schematic diagram of the olfactory epithelium.

Figure 4 shows the mitogenic response (% BrdU-labelled) of dissociated cochlear chick support cells in culture treated with various doses of rhGGF2.

Figure 5 shows the mitogenic response [illegible]

Figure 6 shows neuregulin transcripts detected by *in situ* hybridization in the spiral ganglia of a day 18 mouse embryo.

Figure 7 shows neuregulin transcripts detected by *in situ* hybridization in the vestibular epithelium of a day 21 rat embryo.

Figure 8 shows that rhGGF2 promotes olfactory neuron survival *in vitro*; figure 8A represents 2 days *in vitro* and figure 8B represents 4 days *in vitro*.

Figure 9 shows neuregulin transcripts detected by *in situ* hybridization in the olfactory neuron layer of postnatal rat olfactory epithelium.

DETAILED DESCRIPTION OF THE INVENTION

It is intended that all references cited shall be incorporated herein by reference.

General

The invention pertains to methods of promoting function of non-visual sensory epithelial cells. The function is affected by the administration of a neuregulin to a vertebrate where the neuregulin interacts with a non-visual sensory epithelial cell to promote one or more aspects of non-visual sensory epithelial cell function, including proliferation, differentiation, growth, survival, migration, changes in the pattern of gene expression and secretion, and metabolic change of the non-visual sensory epithelial cell.

Definition of key terms

The term administration as used herein refers to the act of delivering a substance, including but not limited to the following routes: parenteral, intravenous, subcutaneous, intramuscular, intraperitoneal, topical, intranasal, intrathecal, aerosol or oral.

The term affecting as used herein refers to the induction of a quantitative change in the response of a target cell, as a result of an interaction with a neuregulin.

The term basal cell as used herein refers to epithelial cells that reside in immediate

The term differentiation as used herein refers to a morphological and/or chemical change that results in the generation of a different cell type or state of specialization. The differentiation of cells as used herein refers to the induction of a cellular developmental program which specifies one or more components of a cell type. The therapeutic usefulness of differentiation can be seen, in increases in quantity of any component of a cell type in diseased tissue by at least 10% or more, more preferably by 50% or more, and most preferably by more than 100% relative to the equivalent tissue in a similarly treated control animal.

The term disorder as used herein refers to a disturbance of function and/or structure of a living organism, resulting from an external source, a genetic predisposition, a physical or chemical trauma, or a combination of the above, including but not limited to any mammalian disease.

The term erbB receptor as used herein refers to erbB2, erbB3 and erbB4 (also HER-2, HER-3 and HER-4 of human) existing as monomeric, homodimeric and heterodimeric (e.g., erbB2/erbB3) cell surface receptor tyrosine kinases that bind and/or are activated by one or more neuregulins.

The term function as used herein refers to any activity or response of a cell. These include but are not limited to proliferation, differentiation, growth, survival, changes in the pattern of gene expression and secretion, and metabolic changes.

The term hair cell as used herein refers to the sensory cells of the cochlear and vestibular epithelia. Such cells possess stereocilia on their apical surfaces, and the bending of the stereocilia in response to mechanical stimuli (e.g. sound waves) causes signaling by the hair cells.

The term mammal as used herein describes a member of the Class Mammalia (Subphylum Vertebrata).

The term mitosis as used herein refers to the division of a cell where each daughter nucleus receives identical complements of the numbers of chromosomes characteristic of the somatic cells of the species. Mitosis as used herein refers to any cell division which results in the production of new cells in the patient. M[illegible]

[illegible] most preferably 100%, when the cells are exposed to labeling agent for a time equivalent to two doubling times. The mitotic index is the fraction of cells in the culture which have

labeled nuclei when grown in the presence of a tracer which only incorporates during S phase (i.e., BrdU) and the doubling time is defined as the average time required for the number of cells in the culture to increase by a factor of two.

The term neuregulin as used herein refers to the glial growth factors, the heregulins, neu differentiation factor, acetylcholine receptor inducing activity, and erbB2, 3 and 4 binding proteins. A more complete definition of neuregulins can be found in the specification herein and in the following materials: U.S. Patent No. 5,237,056; U.S. Patent Application SN 08/249,322; WO 92/20798; EPO 0 505 148 A1; Marchionni, et al., *Nature* 362:313, 1993; Benveniste, et al., *PNAS* 82:3930, 1985; Kimura, et al., *Nature* 348:257, 1990; Davis and Stroobant, *J. Cell. Biol.* 110:1353, 1990; Wen, et al., *Cell* 69:559, 1992; Yarden and Ullrich, *Ann. Rev. Biochem.* 57:443, 1988; Holmes, et al., *Science* 256:1205, 1992; Dobashi, et al., *Proc. Natl. Acad. Sci.* 88:8582, 1991; Lupu, et al., *Proc. Natl. Acad. Sci.* 89:2287, 1992; Peles and Yarden, *BioEssays* 15:815, 1993, Mudge, *Current Biology* 3:361, 1993, all hereby incorporated by reference.

The term neurological disorder as described herein refers to a disorder of the nervous system.

The term non-visual sensory epithelium as used herein refers to composite of cells and extracellular matrix that comprise the sheet-like layer of tissue in sensory organs that include sensory cells such as hair cells and olfactory neurons. Examples include, but are not limited to the cochlear epithelium, the three vestibular epithelia, the olfactory epithelium and the epithelial layer of the skin. Not included are the sensory epithelium of the visual system, the retina.

The term olfactory neuron as used herein refers to the primary sensory neurons of the olfactory epithelium. These cells project chemosensory cilia into nasal mucosa, and the cilia bind odorant molecules. Odorant binding results in olfactory neuron signaling.

The term support cell as used herein refers to cells residing within epithelia that are not sensory in nature, and that may play supporting functions for the maintenance and regeneration of the epithelium. Such cells may provide trophic support for sensory cells, and may give rise to new sensory cells in the regenerative situation.

of apoptosis or the prevention of other mechanisms of cell loss. Survival as used herein

indicates a decrease in the rate of cell death by at least 10%, more preferably by at least 50%, and most preferably by at least 100% relative to an untreated control. The rate of survival may be measured by counting cells stainable with a dye specific for dead cells (such as propidium iodide) in culture.

The term sustentacular cell as used herein refers to support cells of the olfactory epithelium that reside in the luminal most portion of the epithelium. The cells contribute to the nasal mucosa and abut the chemosensory cilia of the olfactory neurons.

The term therapeutically effective amount as used herein refers to that amount which will produce a desirable result upon administration and which will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

The term treating as used herein may refer to a procedure (e.g. medical procedure) designed to exert a beneficial effect on a disorder. Treating as used herein means any administration of a substance described herein for the purpose of increasing inner ear cell function. Most preferably, the treating is for the purpose of reducing or diminishing the symptoms or progression of a disease or disorder of inner ear cells. Treating or treatment, as used herein, also means the administration of a substance to increase or alter the cells in healthy individuals. The treating may be brought about by the contacting of the cells which are sensitive or responsive to the neuregulins described herein with an effective amount of the neuregulin.

The term vertebrate as used herein refers to an animal that is a member of the Subphylum Vertebrata (Phylum Chordata).

Hair cell regeneration: an approach to therapy

The first evidence for regeneration of mammalian hair cells came from studies of vestibular hair cell regeneration in guinea pigs treated with an ototoxic antibiotic. An accompanying *in vitro* study demonstrated the production of hair cells with an immature phenotype. In both cases, however, any hair cell regeneration might have been unexpected. Support cell division was observed to take place prior to production of new hair cells, but it is evident that certain factors that promote appropriate regeneration are either absent or at low levels in these systems or are inhibited.

Several factors have been identified that may play a role in hair cell production. A candidate factor for a role in hair cell development and regeneration is TGFα, and another is retinoic acid

Retinoic acid has been detected in embryonic and early postnatal mouse cochlea. When placed in culture with exogenous retinoic acid, embryonic mouse cochleas develop supernumerary hair cells in the absence of cell division. This finding suggests that retinoic acid acts as a regulator of differentiation rather than acting as a mitogen for support/precursor cells. Very recently it has been reported that retinoic acid stimulates regeneration of antibiotic-treated postnatal rat cochleas *in vitro*, and that this regeneration is blocked by anti-mitotic agents. In light of the previous retinoic acid result, it appears likely that an endogenous mitogen stimulates division of a hair cell precursor and that retinoic acid stimulates hair cell differentiation.

Neurogenesis and Cell Survival in the olfactory system.

A unique aspect of the olfactory epithelium is that primary olfactory neurons constitute the only population of neurons in all vertebrates that are known to be able to regenerate throughout the lifetime of the animal (Graziadei, et al. *Handbook of Sensory Physiology* 9:55-83, 1977). This ability to regenerate is necessary, because olfactory neurons are directly exposed to airborn pollutants and pathogens, and thus are vulnerable to irreversible damage and death. The regeneration process requires both the production of new olfactory neurons and appropriate targeting of the new axons to the olfactory bulb.

A variety of studies have demonstrated that the basal cells give rise to new olfactory neurons. Early studies in mice (summarized by (Graziadei, et al. *Handbook of Sensory Physiology* 9:55-83, 1977) found that soon after injection of ^{3}H-thymidine, label was observed in basal cells, and subsequently in maturing neurons. Horizontal basal cells take up very little label while globose basal cells take up high levels of ^{3}H-thymidine soon after injection (Monti-Graziadei, et al. *J Neurocytol* 8:197-213, 1979; Miragall, et al. *Brain Res* 239:245-250, 1982). This finding has been confirmed in studies using bromodeoxyuridine (Yamagishi, et al. *Arch Histol Cytol* 52 [Suppl]:375-381, 1989; Suzuki, et al. *Cell Tissue Res* 266:239-245, 1991), and has been interpreted to mean that horizontal basal cells are not precursors of olfactory neurons. Longer term labeling studies, however, reveal a more complex pattern of cell division (Mackay-Sim, et al. *J Neurosci* 11:979-984, 1991). cells lying within one nuclear diameter of the basal lamina displayed a biphasic pattern of labeling. The model proposed to account for the observed pattern of labeling posits a rapid phase of 2-3 successive cell divisions that give rise to migrating immature neurons, and a superimposed slower rate of basal cell division. The fast initial divisions thus correspond to the activity of

Neuregulins

A recently described family of growth factors, the neuregulins (reviewed by Mudge, *Curr. Biol.* 3:361, 1993; Peles and Yarden, *Bioessays* 15:815, 1993), are synthesized by neurons (Marchionni et al. *Nature* 362:313, 1993) and by mesenchymal cells from several parenchymal organs (Mearr and Birchmeier, *PNAS* 91:1064, 1994). The neuregulins and related factors that bind p185^{erbB2} have been purified, cloned and expressed (Benveniste et al. *PNAS*, 82:3930, 1985; Kimura et al., *Nature* 348:257, 1990; Davis and Stroobant, *J. Cell Biol.* 110:1353, 1990; Wen et al., *Cell* 69:559, 1992; Yarden and Ullrich, *Ann. Rev. Biochem.* 57:443, 1988; Dobashi et al., *Proc. Natl. Acad. Sci.* 88:8582, 1991; Lupu et al., *Proc. Natl. Acad. Sci.* 89:2287, 1992; Wen et al., *Mol. Cell. Biol.* 14:1909, 1994). Recombinant neuregulins have been shown to be mitogenic for peripheral glia (Marchionni et al., *Nature* 362:313, 1993) and have been shown to influence the formation of the neuromuscular junction (Falls et al., *Cell* 72:801, 1993; Jo et al., *Nature* 373: 158, 1995; Chu et al., *Cell* 14: 329, 1995).

The neuregulin gene consists of at least thirteen exons. The neuregulin transcripts are alternatively spliced and these encode many distinct peptide growth factors, which are referred to as the neuregulins (Marchionni et al., *Nature* 362:313, 1993). DNA sequence comparisons revealed that neu differentiation factor (NDF) (Wen et al., *Cell* 69:559, 1992) and heregulins (Holmes et al., *Science* 256:1205, 1992), which were purified as ligands of the p185^{erbB2} (also known as neu or HER2) receptor tyrosine kinase, also are splicing variants of the neuregulin gene. The acetylcholine receptor inducing activity (ARIA) also is a product of the neuregulin gene (Falls et al., *Cell* 72:801, 1993). Common structural features of the neuregulins are the presence of a single immunoglobulin-like (Ig) fold and a single epidermal growth factor-like (EGF) domain.

The sites of neuregulin gene expression have been characterized by use of nucleic acid probes to analyze RNA samples by a variety of methods, such as Northern blotting, RNase protection, or *in situ* hybridization. Transcripts have been detected in the nervous system and in a variety of other tissues (Holmes et al., *Science* 256:1205, 1992 Wen et al., *Cell* 69:559, 1992; Mearr and Birchmeier, *PNAS* 91:1064, 1994). Sites of gene expression have been localized in the brain and spinal chord and in other tissues. (Orr-Urteger et al., *PNAS* 90:1867, 1993; Falls et al., *Cell* 72:801, 1993; Marchionni et al., *Nature* 362:313, 1993; Mearr and Birchmeier, *PNAS* 91:1064, 1994). [illegible] neuregulin transcripts has been detected at embryonic day 15 in rat (Mearr and Birchmeier, *PNAS* 91:1064, 1994).

Although a large number of neuregulins may be produced by alternative splicing, they can be broadly sorted into the putative membrane-bound and the soluble isoforms. The former contains a putative trans-membrane domain and may be presented at the cell surface. Membrane-anchored peptide growth factors may mediate cell-cell interactions through cell-adhesion or juxtacrine mechanisms (reviewed by Massagué and Pandiella, *Ann. Rev. Biochem.* 62:515, 1993). Alternatively, the putative membrane-bound isoforms may be cleaved from the cell surface and function as soluble proteins (Wen et al., *Cell* 69:559, 1992; Falls et al., *Cell* 72:801, 1993). The soluble neuregulin isoforms contain sequence corresponding to the extracellular domains of the putative membrane-bound isoforms, but terminate before the transmembrane domain. These neuregulin isoforms may be secreted, and hence could affect cells at a distance; or they may be present in the cytoplasm, but could be released upon cellular injury. In the latter case, neuregulins may function as injury factors, as has been postulated for the ciliary neurotrophic factor (Stöckli et al., *Nature* 342:920, 1989). Any one of these modes of action of the neuregulins may occur in the cochlear-vestibular epithelia.

Cellular targets of peptide growth factors are those which bear receptors for the factor(s) and those that are shown to respond in a bioassay either *in vitro* or *in vivo*. Based on studies demonstrating phosphorylation on tyrosine residues or cross-linking experiments, neuregulins are candidate ligands for the receptor tyrosine kinases $p185^{erbB2}$ (or HER-2 in human), $p185^{erbB3}$ (HER-3 in human), $p185^{erbB4}$ (or HER-4 in human) or related members of the EGFR gene family. Collectively, these receptors can be referred to as erbB receptors. Though the precise ligand-receptor relationship of each neuregulin protein with each member of the EGFR family is yet to be clarified, several lines of evidence suggest that binding of ligands is mediated by either erbB3 and erbB4 , but signaling occurs through either erbB2, erbB4 and heterodimers of the various subunits (e.g., Carraway and Cantley, *Cell* 78:5, 1994). These receptors are known to be present on Schwann cells and muscle cells (Jo et al., *Nature* 373: 158, 1995), and other neuregulin targets, such as cell lines derived from various tumor tissues, such as breast and gastric epithelia. Sites of expression of the HER-4 gene have been localized by *in situ* hybridization to several regions of the brain, including: hippocampus, dentate gyrus, neo cortex, medial habenula, reticular nucleus of the thalamus, and the amygdala (Lai and Lemke, *Neuron* 6:691, 1991). The distribution of the HER-4 receptor has not been studied by methods that allow detection of the protein or the activated receptor tyrosine kinase *in vivo* or in cultures of primary cells. The expression pattern of

Neuregulins have been shown to have a variety of biological activities depending on the cell type being studied. Several neuregulins, including native bovine GGFI, II and III and recombinant human GGF2 (rhGGF2) are mitogenic for Schwann cells (Marchionni et al., *Nature* 362:313, 1993), as is heregulin B1 (Levi et al, *J Neurosci.* 15:1329, 1995). On human muscle culture, rhGGF2 has a potent trophic effect on myotubes (Sklar et al., U.S. Pat. Applic. # 08/059, 022). The differentiation response to rhGGF2 also includes induction of acetylcholine receptors in cultured myotubes (Jo et al., *Nature* 373: 158, 1995). This activity is associated with other forms of neuregulin, including ARIA (Falls et al., *Cell* 72:801, 1993) and heregulin B1 (Chu et al., *Neuron* 14:329, 1995), as well as with rhGGF2. Further, ARIA has been shown to induce synthesis of voltage-gated sodium channels in chick skeletal muscle (Corfas and Fischbach, *J. Neurosci.* 13:2118, 1993). Glial growth factor (GGF), and more specifically rhGGF2, can restrict neural crest stem cells to differentiate into glial cells *in vitro* (Shah et al., *Cell* 77:349, 1994). Activities of neuregulin on inner ear cells have not been described. In summary, there are examples of neuregulin proteins affecting proliferation, survival and differentiation of target cells.

A novel aspect of the present invention relates to the ability of neuregulins to affect function of inner ear cells and olfactory epithelial cells. Neuregulins are the products of a gene which produce a number of variably-sized, differentially-spliced RNA transcripts that give rise to a series of proteins. These proteins are of different lengths and contain some common peptide sequences and some unique peptide sequences. The conclusion that these factors are encoded by a single gene is supported by the differentially-spliced RNA sequences which are recoverable from bovine posterior pituitary, human spinal chord and human breast cancer cells (MDA-MB-231). Further support for this conclusion derives from the size range of proteins which act as ligands for the erbB receptors (see below).

Further evidence to support the fact a single gene encodes the various neuregulins derives from nucleotide sequence comparisons. Holmes et al., (*Science* 256:1205, 1992) demonstrate the purification of a 45-kilodalton human protein (Heregulin-α) which specifically interacts with the receptor protein p185erbB2. Peles et al., (*Cell* 69:559, 1992) describe a complementary DNA isolated from rat cells encoding a protein call "neu differentiation factor" (NDF). The translation product of the NDF cDNA has p185erbB2 binding activity. Several other groups have reported the purification of proteins of various molecular weights with erbB receptor binding activity. These groups include the following: Lupu et al., *Proc. Natl. Acad. Sci. USA* 89:2287, 1992; Yarden and Peles, *Biochemistry*

We have found that proteins that bind p185erbB2 and related receptors (i.e., p185erbB3 and p185erbB4) affect inner ear cell mitogenesis (Examples 1 and 2). Further, the presence of neuregulin transcripts (Example 3 and 4) in inner ear cells *in vivo* indicates that neuregulin has a role in inner ear cell function *in vivo*. We also have found that neuregulins affect survival of olfactory cells (Example 5) and that neuregulins transcripts are detected in cells of the olfactory epithelium (Example 6).

These neuregulins may be identified using the protocols described herein and in Holmes et al., *Science* 256: 1205, 1992; Peles et al., *Cell* 69:205, 1992; Wen et al., *Cell* 69:559, 1992; Lupu et al., *Proc. Natl. Acad. Sci. USA* 89:2287, 1992; Yarden and Peles, *Biochemistry* 30:3543, 1991; Lupu et al., *Science* 249:1552, 1990; Dobashi et al., *Biochem. Biophys. Res. Comm.* 179:1536, 1991; Huang et al., *J. Biol. Chem.* 257:11508-11512, 1992; Marchionni et al., *Nature* 362:313, 1993; and in U.S. Patent Application Serial No. 07/931,041, filed August 17, 1992, and U.S. Patent No. 5,530,109 issued on June 25, 1996, all of which are incorporated herein by reference.

Compounds can be assayed for their usefulness *in vitro* using the methods provided in the description and examples below. Following the *in vitro* demonstration of the effect of neuregulins on inner ear cell function, the *in vivo* therapeutic benefit of the effect can be accomplished by the administration of neuregulins, neuregulin producing cells or DNA encoding neuregulins to a vertebrate requiring therapy.

In Vitro Assays of Neuregulin Effects on Non-visual Sensory Epithelial Cells

Several *in vitro* assays are used to determine which neuregulin protein(s) promote non-visual sensory epithelial cell function and which non-visual sensory epithelial cell types are affected by contacting neuregulin protein. Described below are methods for detecting the ability of a neuregulin to promote function of a non-visual sensory epithelial cell. *In vitro* assays for determining neuregulin effects on non-visual sensory epithelial cell function depend on establishing non-visual sensory epithelial cell cultures. A general reference on cell and tissue culture is *Cell and Tissue Culture: Laboratory Procedures* (Ed. by A. Doyle, J. B. Griffiths, and D. G. Newell, John Wiley and Sons, New York, NY, 1994). General references on the culture of neural cells and tissues are *Methods in Neurosciences, Vol. 2* (Ed. by P. M. Conn. Academic Press, Sand Diego, CA, 1990) and *Culturing Nerve Cells* (Ed. by G. Banker and K. Goslin, MIT Press, Cambridge, MA, 1991). General references

Immunocytochemistry II (Ed. by A. C. Cuello, John Wiley and Sons, New York, NY, 1993).

The non-visual sensory epithelial cell from a vertebrate used in this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing non-visual sensory epithelial cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.* 58:44, 1979; Barnes and Sato, *Anal. Biochem.* 102:255, 1980; U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195, may be used as culture media for non-visual sensory epithelial cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, will be apparent to the ordinarily skilled artisan.

The use of non-visual sensory epithelial cell cultures to demonstrate that neuregulin promotes non-visual sensory epithelial cell function is in accordance with the methods described in general terms above and further described below.

***In Vitro* Method I**

The survival function is assayed by methods that identify and count either viable cells or dead non-visual sensory epithelial cell cells following culture at low density (e.g., 10,000 cells/cm^2) over a period from one to six days in the presence of varying amounts of neuregulin added to the culture medium. Included in these methods are specific stains for dead cells, such as propidium iodide, which enters the nucleus of dead cells and is detected by fluorescence microscopy. Alternatively, the counting of non-visual sensory epithelial cells adhering to the culture substratum over a six day period also can be used as an indicator of cell survival.

***In Vitro* Method II**

An alternative procedure to monitor non-visual sensory epithelial cell death utilizes labeling of nicked DNA strands, which are characteristic of cells undergoing apoptotic cell death, with digoxygenin-11-dUTP using terminal deoxynucleotidyl transferase (TUNEL) according to the protocol described in Gavrieli et al., *J. Cell Biol.* 119: 493-501, 1992. The labeled DNA strands are detected using standard kits available from commercial vendors (e.g., Genius kit from Boehringer Mannheim). Further, a cell death detection ELISA system, which is based on the DNA fragmentation that occurs in dying cells (Boehringer Mannheim catalog no. 1585 045) can be utilized to quantify cell death in accordance with the instructions provided by the commercial vendor.

***In Vitro* Method III**

The release into the culture medium of the cytosolic enzyme lactate dehydrogenase (LDH) also can be used to quantify the extent of non-visual sensory epithelial cell death *in vitro* (Kirk et al, *J. Pharmacol. Exper. Therapeut.* 271:1080, 1994). LDH levels are measured by an automated kinetic colorimetric assay in which oxidation of lactate to pyruvate is coupled to reduction of the tetrazolium dye, INT. Briefly, 80 ul samples of the culture medium are mixed with an equal volume of the substrate solution containing (in mg/l) INT, 334; phenazine methosulfate, 86; nicotinamide adenine dinucleotide, 862; L-(+)-lactate, 4900 (lithium salt); and 0.1% Triton X-100 in 0.2 M Tris buffer, pH 8.2. In the assay, LDH activity is directly proportional to the rate of appearance of the resulting INT formazan (absorbance max. at 492 nm). The product is monitored quantitatively in a microplate reader (UVmax, Molecular Devices, Menlo Park, CA) as the change in absorbance at 490 nm over a 2 minute interval.

***In Vitro* Method IV**

The proliferative function of neuregulins on non-visual sensory epithelial cells can be assayed by incorporation of ^{125}I-Urd, ^{3}H-dT or BrdU into replicating DNA strands of dividing cells, or by cell counting. The assays developed to measure the mitogenic activity of neuregulins on Schwann cells by incorporation of DNA synthesis precursors (Brockes et al., *Brain Res.* 165:105, 1979; Davis and Stroobant, *J. Cell Biol.* 110: 1353, 1990) can be adapted to non-visual sensory epithelial cells by one of normal skill in the art of cell culture.

***In Vitro* Method V**

The differentiation function of neuregulins on non-visual sensory epithelial cells can be assayed by employing analytical methods, such as staining of filamentous actin of hair cell stereocilia and cuticular plates by fluorescently conjugated phalloidin, immunostaining of *in situ* hybridization, which can detect and quantify marker proteins associated with the various cell types of non-visual sensory epithelia, such as myosin 7, osteospondin, hair cell antigens (HCA), calbindin, tectorins, and other antigens in hair cells and supporting cells of the cochlear-vestibular epithelia which are specifically bound by monoclonal antibodies.

***In Vitro* Method VI**

Several peptide growth factors and their receptors have been identified in non-visual sensory epithelia, as described in the prior act. Methods utilized to detect those molecules and activities can be employed to demonstrate a differentiation function of neuregulin on non-visual sensory epithelial cells. For example, neuregulins can be shown to induce the synthesis of growth factors and/or their receptors expressed in the cochlear-vestibular epithelia. The analysis can be by *in situ* hybridization or other methods of quantitative RNA analysis, such as, but not limited to, reverse transcription-PCR, RNAse protection and Northern blotting. Alternatively, induced expression of growth factors or their receptors can be assayed by immunocytochemical staining or cell biological assays designed to measure growth factor activity.

The *in vitro* assays described above to identify neuregulins that have biological activity on non-visual sensory epithelial cells can be applied to dissociated cells, semi-dissociated cells, explants of whole non-visual sensory epithelia and parts thereof, such as preparations of the cochlear-vestibular epithelia or of the olfactory epithelia. The cultures can be established and maintained using methods described above. In some cases, minor modifications or substitutions to the procedures described herein, which do not alter the reduction to practice of the invention, can be provided by one of ordinary skill in the art.

***In Vivo* Assays of Neuregulin Effects on non-visual sensory epithelial cells.**

Neuregulin activity on inner ear cells also can be shown through *in vivo* assays. Some

***In Vivo* Method I**

Hair cell regeneration has been studied *in vivo* in a variety of vertebrate species. A general model for the process as described in the avian cochlea is outlined below.

Damage to cochlear epithelia is brought on by acoustic trauma. Ototoxic antibiotics can be used to damage both the cochlear and the vestibular epithelia. Regeneration of chick sensory hair cells after acoustic trauma illustrates two key points: 1) the perturbations replicate the manner in which human hearing loss occurs, and; 2) regeneration of the damaged tissue leads to restoration of hearing function over a period of days to weeks. Since the lost neuroepithelial cells are part of a local neural circuitry the re-establishment of appropriate synaptic connections is quite feasible and has been demonstrated in this model and in other models of hair cell regeneration.

Acoustic overstimulation for 48 hours at 1.5 kHz leads to severe damage to hair cells in the cochlea (Corwin and Cotanche, *Science* 240: 1772-1774, 1988). The damage to the cochlea and the recovery process can be monitored by several techniques, including microscopy and tests for hearing function or impairment. Regions of the epithelia located at a point approximately 30% of the distance along the epithelium from the proximal end in a cochlea (that is fixed immediately after 48 hours of acoustic overstimulation at 1.5 kHz) can be seen to have hair cell stereocilia bundles missing in the area damaged by the sound. Normal stereocilia bundles surround the lesion. Regeneration that occurs in the cochlear sensory epithelium of chickens can be observed by utilizing scanning electron microscopy. A chick cochlear epithelium fixed 10 days after acoustic trauma shows that nearly normal appearance has been restored. Similar models are available in rat and guinea pig, where hair cell regeneration in vestibular end organs is much more limited. The key to promoting mammalian hair cell regeneration would seem to lie in identifying and delivering the appropriate stimulus for proliferation of support cells and differentiation or repair of hair cells. Neuregulins delivered to the damaged epithelial tissue can stimulate proliferation of support cells in the otic neuroepithelium (cochlear and vestibular) and thus promote the regeneration of hair cells. Thus, neuregulin administered to the lesion site in an therapeutically effective amount can be shown to promote the process of hair cell regeneration leading to the recovery of inner ear function of hearing and balance.

***In Vivo* Method II**

[illegible] consecutive days to traumatize both the vestibular and cochlear sensory epithelium. Dorge

et al. *Science* 259: 1616-1619, 1993). Following this treatment animals are fitted with osmotic micropumps to deliver either vehicle alone or rhGGF2 in vehicle directly to the perilymphatic fluid compartments of the inner ear via a cannula. Some groups of animals (10 animals/group) are sacrificed at 7-10 days to analyze the level of proliferation of support cells, while other groups are allowed to continue recovery for 28 to 60 days to assess hair cell regeneration. Labelling with bromodeoxyuridine, also delivered via osmotic micropumps, is used to monitor proliferation in whole mount and sectioned preparations, and hair cell regeneration can be assessed by microscopy.

***In Vivo* Method III**

Bulbectomy (based on M. Schwartz Levey, D.M. Chikaraishi, and J.S. Kauer, *J. Neurosci.* 11:3556-3564 1991):

Male mice, aged 3 months, are used for these experiments. Olfactory bulbectomy is performed on the right side of each animal under sterile conditions, the left side serving as a control. Under Avertin anesthesia (tribromoethanol and amyl alcohol, warmed to 37° C), the overlying skull is opened and the right olfactory bulb is removed by aspiration. A piece of Gelfoam is placed in the bulb site to prevent bleeding. By 3 days, the olfactory epithelium on the experimental side starts to show structural changes accompanied by neuronal degeneration. By 7 days post-lesion, newly formed epithelial cells can begin to be detected. By 14 days post-lesion, a nearly normal olfactory epithelium has been regenerated.

One could inject rhGGF2 systemically and/or apply it topically, at various times and doses and look for either neuroprotection and/or the acceleration of regeneration.

***In Vivo* Method IV**

Partial olfactory nerve lesion (based on M. Cagiano, J.S. Kauer, and D.D. Hunter, *Neuron* 13:339-352, 1994):

Rat pups, 2-5 days old, are anesthetized, and the experimental animals are lesioned at the level of the cranial extension of the olfactory nerve, by penetration through the cartilage with a blunt needle inserted just caudal to the cribriform plate. This resultant damage to the olfactory nerve causes degeneration of part of the olfactory epithelium.

look for either neuroprotection and/or the acceleration of regeneration.

Other compounds, in particular peptides, which specifically bind and/or activate erbB receptors also can be used according to the invention as effectors of non-visual sensory epithelial cell function. A candidate compound can be routinely screened for erbB receptor binding, and if it binds, can then be screened for affecting inner ear cell function using the methods described herein.

The demonstration of biological activity of the neuregulins by promoting inner ear cell function in any of the animal models described above indicates efficacy in treating disorders of the ear. A variety of inner ear diseases and related disorders are known that produce impaired hearing and/or balance and in some cases progress to total deafness. These disorders of the ear include, but are not necessarily limited to: sensorineural hearing loss caused by ototoxic antibiotics, and by chemotherapeutic agents such as cisplatin which produces a peripheral neuropathy resulting in hearing loss, excessive noise, viral and bacterial infection or autoimmune disease; tinnitus; and, Meniere's syndrome. Meningitis often results in hearing loss. Thus, administration of neuregulin in a therapeutically effective amount can provide a treatment for disorders of the ear, which otherwise left untreated would result in the loss of hearing and/or balance.

The invention includes use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity related to affecting inner ear cell function and more generally to non-visual sensory epithelial cell function. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The invention includes the use of the above named family of proteins (i.e. neuregulins) as extracted from natural sources (tissues or cell lines) or as prepared by recombinant means.

The human peptide sequences described above represent a series of splicing variants which can be isolated as full length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

homologous to coding segments which comprise neuregulin, as well as other naturally

occurring neuregulin polypeptides for the purpose of promoting inner ear cell function. Also included are the use of: allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes under high or low stringency conditions to a nucleic acid naturally occurring (for definitions of high and low stringency see *Current Protocols in Molecular Biology*, (1989) John Wiley & Sons, New York, NY, 6.3.1 - 6.3.6, hereby incorporated by reference); and the use of polypeptides or proteins specifically bound by antisera to GGF polypeptides. The term also includes the use of chimeric polypeptides that include the neuregulin polypeptides.

Use of Neuregulins

A novel aspect of the invention involves the use of neuregulins as factors to promote non-visual sensory epithelial cell function. Treatment of the cells to achieve these effects may be achieved by contacting cells with a polypeptide described herein.

The methods of the invention make use of the fact that the neuregulin proteins are encoded by the same gene. A variety of messenger RNA splicing variants (and their resultant proteins) are derived from this gene and many of these products show binding to erbB receptors and activation of the same. This invention provides a use for all of the known products of the neuregulin gene (described herein and in the references listed above). Most preferably, recombinant human GGF2 (rhGGF2) is used in these methods.

The invention includes use of any modifications or equivalents of the above polypeptide factors which do not exhibit a significantly reduced activity. For example, modifications in which amino acid content or sequence is altered without substantially adversely affecting activity are included. The statements of effect and use contained herein are therefore to be construed accordingly, with such uses and effects employing modified or equivalent factors being part of the invention.

The human peptide sequences described above represent a series of splicing variants which can be isolated as full-length complementary DNAs (cDNAs) from natural sources (cDNA libraries prepared from the appropriate tissues) or can be assembled as DNA constructs with individual exons (e.g., derived as separate exons) by someone skilled in the art.

respectively, optionally together with an acceptable diluent, carrier or excipient and/or in unit

dosage form. In using the factors of the invention, conventional pharmaceutical or veterinary practice may be employed to provide suitable formulations or compositions.

A medicament is made by administering the polypeptide with a pharmaceutically effective carrier. Neuregulins can be administered into the perilymphatic fluid compartments of the inner ear by injection of factor formulated in an appropriate vehicle for administration. An osmotic mini-pump may also used to deliver neuregulin directly to the perilymphatic fluid compartments of the inner ear via a cannula. There is also the option of delivering the factor using ethylene-vinyl acetate copolymer implants or by delivery to the cerebral spinal fluid by intrathecal injection.

Thus, the formulations to be used as a part of the invention can be applied to parenteral administration, for example, intravenous, subcutaneous, intramuscular, intraperitoneal, topical, intranasal, intrathecal, aerosol, transdermal and by other slow release devices (i.e., osmotic pump-driven devices; see also USSN 08/293,465, hereby incorporated by reference)

The formulations of this invention may also be administered by the transplantation into the patient of host cells expressing the DNA encoding polypeptides which are effective for the methods of the invention or by the use of surgical implants which release the formulations of the invention.

Parenteral formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations maybe in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well-known in the art for making formulations are to be found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain as excipients sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes, biocompatible, biodegradable lactide polymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the present factors. Other potentially useful parenteral delivery systems for the factors include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for [illegible] glycocholate for buccal administration, methoxysalicylate for rectal administration, or citric acid for vaginal administration

The present factors can be used as the sole active agents, or can be used in combination with other active ingredients, e.g., other growth factors which could facilitate neuronal survival in neurological diseases, or peptidase or protease inhibitors.

The concentration of the present factors in the formulations of the invention will vary depending upon a number of issues, including the dosage to be administered, and the route of administration.

In general terms, the factors of this invention may be provided in an aqueous physiological buffer solution containing about 0.1 to 10% w/v compound for parenteral administration. General dose ranges are from about 1 µg/kg to about 1g/kg of body weight per day; a preferred dose range is from about 0.01 mg/kg to 100 mg/kg of body weight per day. The preferred dosage to be administered is likely to depend upon the type and extent of progression of the pathophysiological condition being addressed, the overall health of the patient, the make up of the formulation, and the route of administration.

A further general aspect of the invention is the use of a factor of the invention in the manufacture of a medicament, preferably for the treatment of a disease or disorder. The "GGF2" designation is used for all clones which were previously isolated with peptide sequence data derived from GGF-II protein (i.e., GGF2HBS5, GGF2BPP3) and, when present alone (i.e., GGF2 OR rhGGF2), to indicate recombinant human protein encoded by plasmids isolated with peptide sequence data derived from the GGF-II protein (i.e., as produced in insect cells from the plasmid HBS5). Recombinant human GGF from the GGFHBS5 clone is called GGF2, rhGGF2 and GGF2HBS5 polypeptide.

Methods for treatment of diseases or disorders using nucleic acid constructs encoding neuregulins or neuregulin producer cells are also part of the invention.

Delivery of DNA to a cell or tissue that will take up the DNA, express the DNA and produce neuregulin as shown by Wolff et al., (*Science* 247:1465, 1990) and Ascadi et al., (*Nature* 352:815, 1991) is an aspect of the invention. Genetic modification of cultured cells (or their precursors) such as fibroblasts (as shown by Wolff et al. *Proc. Nat'l Acad. Sci. USA* 86:1575, 1988) or such as those derived from the nervous system (as shown by Weiss et al. International Patent Application number PCT/US94/01053, publication number WO

[illegible] genetically modified neuregulin producer cells can be transplanted to a position near the inner ear cell type and elicit the responses described above.

As will be seen from Examples 1 and 2, below, the present factors exhibit mitogenic activity on inner ear cells. In addition to stimulating support cell division, other possible roles for neuregulin/rhGGF2 might include induction of hair cell regeneration, promotion of hair cell-neuron synaptogenesis, and maintenance of both hair cell survival and synaptic function. Also, the present factors exhibit survival activity on olfactory cells. The general statements of invention above in relation to formulations and/or medicaments and their manufacture should clearly be construed to include appropriate products and uses.

A series of experiments follow which provide additional basis for the claims described herein. The following examples relating to the present invention should not be construed as specifically limiting the invention, or such variations of the invention, now known or later developed.

The examples illustrate our discovery that recombinant human GGF2 (rhGGF2) confers effects on non-visual sensory epithelial cells. These activities indicate efficacy of GGF2 and other neuregulins in inducing wound repair and repair of other non-visual sensory epithelial tissue damage, and promoting regeneration and prophylactic effects on non-visual sensory epithelial tissue degeneration.

EXAMPLES

The following examples are designed to illustrate certain aspects of the present invention. The examples are not intended to be comprehensive of all embodiments of the present invention, and should not be construed as limiting the claims presented herein.

Example 1: Neuregulin (rhGGF2) promotes mitogenesis of chick hearing organ epithelia *in vitro*.

Dissociated chick cochlea supporting cells were plated onto fibronectin coated well in Medium-199 with 20% Fetal Bovine Serum. After 96 hours, the serum containing media was removed and cells were maintained in serum free M-199 with N2 supplement for 24 hours. M-199/N2 containing 0-200 ng/ml rhGGF2 was then added to the cells for 24 hours with BrdU added for the last 4 hours. Cells were then fixed and processed for BrdU immunohistochemistry using fluorescent double labeling techniques with BrdU labeled nuclei

In the dissociated cell culture of the avian cochlea (data shown in Figure 4) the basal level of the support cell proliferation is 12.1 %, and therefore, the effect of rhGGF2 appears to be to produce roughly a doubling in the percent labelled cells over the basal level.

Example 2: Neuregulin (rhGGF2) promotes mitogenesis of rat balance organ epithelia *in vitro*

Sensory epithelia were isolated from rat utricle and plated onto fibronectin coated wells in DMEM/F12 with 10% Fetal Bovine Serum. After 8 days, medium was removed and fresh media added containing 12.5 to 100 ng/ml rhGGF2. After 72 hours, BrdU was added to the cultures for 48 hours. Cells were then fixed and processed for BrdU immunohistochemistry using fluorescent double labeling techniques with BrdU labeled nuclei fluorescing red and unlabeled nuclei fluorescing green. The data in Figure 5 show the number of BrdU labeled nuclei found in each culture. The mitogenic stimulation by rhGGF2 is roughly twenty-fold over the basal level at 50 ng/ml and nearly eight-fold over the basal level at 12.5 ng/ml. For comparison, TGFα produces little more than a doubling of the basal rate of proliferation in cultures of mouse vestibular epithelia.

Example 3: Neurons of the spiral ganglion express high levels of neuregulin.

Neurons of the spiral ganglion innervate and make synapses with hair cells of the cochlear epithelium (ce) (see also Figure 2). Ten micron paraffin sections of the embryonic day X rat incubated with a single-stranded ^{35}S-labeled riboprobe (antisense strand) encoding the EGF-like domain through the cytoplasmic domain of the rat cDNA clone GGFRP3 (Marchionni et al., *Nature* 362:312, 1993), show (Figure 6) that neuregulin message is normally expressed in the developing spiral ganglion (sg), and thus that products of the neuregulin gene are normally expressed in a site where they may affect a hair cell epithelium. The survival of spiral ganglion neurons depends on the expression of trophic factors BDNF and NT3 from cells in the sensory epithelia. Neuregulin growth factors expressed in the neurons of the spiral ganglia can elicit the expression of the neurotrophins NT3 and BDNF from cells in the sensory epithelia as part of a trophic support circuit (Gywnne et al., U.S. Patent Application# 08/341,018: Use of neuregulins as modulators of cellular communication) and thus stimulate hair cell regeneration and promote recovery of hearing loss.

Example 4: Epithelial cells of the vestibular system express high levels of neuregulin.

The vestibular epithelium (ve) is the hair cell epithelium of the vestibular system, and thus mediates the sense of balance. Using ten micron frozen sections incubated with a single-stranded digoxigenin-labeled riboprobe (antisense strand) encoding the EGF-like domain through the cytoplasmin domain of the rat cDNA clone GGFRP3 (Marchionni et al., *Nature* 362:312, 1993), we show that (Fig. 7) neuregulin message is normally expressed in the embryonic day 21 rat vestibular system. Neuregulin transcripts are not detected in the non-sensory epithelium (nse).

Example 5: Recombinant human GGF2 promotes olfactory neuron survival in vitro.

All reagents were from Sigma unless otherwise noted. The defined medium used for the culture of primary olfactory neurons, NSFM, is a modification of NYSF-404 (Yabe, et al. *In vitro* 22:363-368, 1986): A 2x stock of basal medium was produced by dissolving the contents of 1 vial of MEM (Sigma Cat. #M-0643) and 1 vial of RPMI-1640 (Sigma Cat. #R-6504) (each meant for 1 liter) into 1 liter of tissue culture-grade water. To 250 ml of stock was added 200 ml water, 5 ml amino acid mix (AAM, see below), 0.5 ml B-vitamin/putrescine mix (BVP, see below), 2 ml 2.5 mg/ml insulin in 5 mM HCl, 0.5 ml 10 mg/ml transferrin in PBS, 10 ml 5% bovine serum albumin (BSA) in PBS, 0.25 g glucose, 0.5 ml 1.7 µg/ml sodium selenate, 5 ml 100 mM sodium pyruvate , 20 µl ethanolamine, 0.7 g sodium bicarbonate, 5 ml of 10,000 U/ml penicillin-10 mg/ml streptomycin, and brought up to a final volume of 500 ml with water. AAM was made by adding 0.15 g of each of the following, except where noted, to 100 ml of water, and stored at -20° in 5 ml aliquots: Arg, Asp, Gln (3 g), Gly (0.05 g), Pro (0.05 g), Ser, Thr, Val, and choline chloride (0.25 g). BVP was made by dissolving 0.0125 g vitamin B-12, 0.025 g biotin, and 0.125 g putrescine into 10 ml water; this was diluted 1000-fold and stored as 0.5 ml aliquots at -20°. Recombinant human GGF2 was stored at -80°C, and diluted into NSFM at the indicated concentrations.

Cultures were produced by a simplification of the protocol of Calof and Lander (Calof, et al. *J Cell Biol* 115:779-794, 1991). Three to four day old rat pups were sacrificed individually, the head bisected with a sagittal cut, the nasal septum removed, and

[illegible] PBS [illegible]

20-25 rats were then coarsely minced with iridectomy scissors, placed in a 15 ml centrifuge

tube with 10 ml of Ca^{++}/Mg^{++}-free HBSS (CMF-HBSS) (Gibco) containing 1 mg/ml trypsin (Type XIII) and 1 mg/ml collagenase (Type A, Boehringer Mannheim), and rotated end-over-end at 37°. After 20 min, the tissue was triturated 5-10 times with a 5 ml tissue culture pipet, and returned to 37° for 25 min. All bony material and undigested tissue was allowed to settle out for approximately 2-3 min at 1 g, the supernatant transferred to a new 15 ml tube, and centrifuged at 100 g for 5 min in a benchtop centrifuge (IEC Centra-7) with the brake turned off. The pellet was resuspended with a flame-polished Pasteur pipet in 10 ml of CMF-HBSS containing 1 mg/ml trypsin inhibitor (Type II-0, from chicken egg white) and 0.5 mg/ml deoxyribonuclease I (DNase), and returned to the warm room for 20 min. The tube was then placed in the centrifuged with the brake turned off, the power turned on for the amount of time necessary to reach 5 g, and immediately turned off; this spin lasted approximately 10 min and allowed the selective pelleting of semi-dissociated pieces of OE sheet. In order to reduce fibroblast number, the pellet was resuspended in 15 ml of NSFM, and the OE tissue pre-plated in 3, 60 mm polystyrene Petri dishes for 2-3 hr at 37° in a 5% CO_2 incubator.

Purified pieces of semi-dissociated OE do not adhere to Petri plastic, and were collected by repeating the very low speed centrifugation. Final plating in NSFM with the indicated factors resulted in cultures referred to as "semi-dissociated." Alternatively, the semi-dissociated pieces were collected after pre-plating, incubated in 2 ml of CMF-HBSS with 1 mg/ml trypsin and 1 mM EGTA for 10 min in a 37° water bath, and triturated to single cells with a flame-narrowed Pasteur pipet. To this was added 5 ml of CMF-HBSS with 2.5 mg/ml trypsin inhibitor and 1 mg/ml DNase, the cells incubated at 37° a further 10 min, and the pellet resuspended for final plating by centrifugation at 100 g for 10 min. Resultant cultures are referred to as "fully dissociated." On average, 7.5×10^5 viable cells (as judged by Trypan blue exclusion) were plated per 35 mm dish.

To prepare culture substrates, 22 mm^2 glass coverslips or regular glass microscope slides were washed serially for 30 min in 4M NaOH, 10 min in deionized water (3 times), 5 min in 100% ethanol, and allowed to dry completely under UV illumination in a laminar-air flow tissue culture hood. For the daily tracking of identified cells/clusters, microscope slides were inscribed with a grid using a diamond-tip scribe prior to cleaning. The cleaned glass was then incubated at 37°, overnight with a coating of 25 µg/ml fibronectin (from bovine plasma) in PBS: 100 µl/coverslip, and 200 µl/slide. It was found that rat OE derived cells do not adhere to laminin. For most cultures, the OE tissue derived from 20-25 rat pups was plated on 50 coverslips, or 6-10 slides.

See also Figure 8. mOGF promotes olfactory neuron survival *in vitro*. When assessed at 2 days *in vitro*, doses of 50 ng/ml or higher give a maximal increased in survival

over control cultures (approximately 2.8-fold increase). By 4 days *in vitro*, a time at which very few neurons survive in serum-free medium, the presence of rhGGF2 at 100 ng/ml gives rise to a approximately 10-fold increase in the number of surviving neurons.

Example 6: Olfactory neurons express high levels of neuregulin.

It has previously been reported that neuregulins are expressed in the olfactory epithelium of embryonic mice using in situ hybridization. This hybridization, however, was performed using ^{35}S-labeled probes and the resolution was such that the cell types expressing neuregulin could not be identified (Meyer, et al. *Proc Natl Acad Sci USA* 91:1064-1068, 1994). Using ten micron paraffin sections incubated with a single-stranded digoxigenin-labeled riboprobe (antisense strand) encoding the EGF-like domain through the cytoplasmin domain of the rat cDNA clone GGFRP3 (Marchionni et al., *Nature* 362:312, 1993), we show (Fig. 9) that neuregulin message is normally expressed in the postnatal rat olfactory epithelium, and this message is expressed within the olfactory neuron layer (onl) and not in the sustentacular cell layer (scl).

CLAIMS

1. A method for treating non-visual sensory epithelial cells of a mammal comprising administration to said mammal of a therapeutically effective amount of a polypeptide which binds an erb B receptor.

2. A method of claim 1, wherein said polypeptide which binds an erb B receptor comprises glial growth factor 2.

3. A method of claim 1, wherein said polypeptide which binds an erb B receptor comprises recombinant human glial growth factor 2.

4. A method for treating non-visual sensory epithelial cells of a mammal comprising administration to said mammal of a DNA sequence encoding a polypeptide which binds an erb B receptor.

5. A method for treating non-visual sensory epithelial cells of a mammal, said method comprising contacting said non-visual sensory epithelial cells with a therapeutically effective amount of a polypeptide which activates an erb B receptor.

6. A method for the prophylaxis of a pathophysiological condition of non-visual sensory epithelial cells in a mammal in which said condition involves a non-visual sensory epithelial cell type which is sensitive or responsive to a polypeptide which binds an erb B receptor, said method comprising administering a therapeutically effective amount of said polypeptide which binds an erb B receptor.

7. A method for the treatment of a pathophysiological condition of non-visual sensory epithelial cells in a mammal, wherein said condition involves a non-visual sensory epithelial cell type which is sensitive or responsive to a polypeptide which binds an erb B receptor, said method comprising administering a therapeutically effective amount of said polypeptide which binds an erb B receptor.

8. A method for the treatment of a condition which involves non-visual sensory epithelial cell damage in a mammal, said method comprising contacting said non-visual sensory epithelial cells with an effective amount of a neuregulin polypeptide

9. The method of claim 7, wherein said treatment of the pathophysiological condition of non-visual sensory epithelial cells results in decreasing the atrophy of said non-visual sensory epithelial cells.

10. The method of claim 7, wherein said treatment of the pathophysiological condition of non-visual sensory epithelial cells results in increasing the non-visual sensory epithelial cells present in said mammal.

11. The method of claim 7, wherein said treatment of the pathophysiological condition of non-visual sensory epithelial cells results in increasing the non-visual sensory epithelial cell survival in said mammal.

12. A method of claim 7, wherein said pathophysiological condition of non-visual sensory epithelial cells is sensorineural hearing loss.

13. A method of claim 7, wherein said pathophysiological condition of non-visual sensory epithelial cells is sensorineural loss of balance.

14. A method of claim 7, wherein said pathophysiological condition of non-visual sensory epithelial cells is sensorineural hearing loss caused by ototoxic antibiotics.

15. A method of claim 7, wherein said pathophysiological condition of non-visual sensory epithelial cells is sensorineural hearing loss caused by excessive noise.

16. A method of claim 7, wherein said pathophysiological condition of non-visual sensory epithelial cells is sensorineural hearing loss caused by viral infection.

17. A method of claim 7, wherein said pathophysiological condition of non-visual sensory epithelial cells is sensorineural hearing loss caused by an autoimmune disease.

18. A method of claim 7, wherein said pathophysiological condition of non-visual sensory epithelial cells is sensorineural hearing loss caused by tinnitus.

19. A method as claimed in claim 7, wherein said pathophysiological condition involves non-visual sensory epithelial cell damage.

21. A method of claim 7, wherein said non-visual sensory epithelial cell type is a vestibular hair cell.

22. A method of claim 7, wherein said non-visual sensory epithelial cell type is an auditory support cell.

23. A method of claim 7, wherein said non-visual sensory epithelial cell type is a vestibular support cell.

24. A method of claim 7, wherein said non-visual sensory epithelial cell type is a sustenactular cell.

25. A method of claim 7, wherein said non-visual sensory epithelial cell type is a basal cell.

26. A method of claim 7, wherein said non-visual sensory epithelial cell type is a Meissner's corpuscle.

27. A method of claim 7, wherein said non-visual sensory epithelial cell type is a Pacinian corpuscle.

28. A method for the prophylaxis or treatment of a pathophysiological condition of non-visual sensory epithelial cells in a vertebrate, wherein said condition involves a non-visual sensory epithelial cell type which is sensitive or responsive to a neuregulin polypeptide, said method comprising administering to said vertebrate a therapeutically effective amount of said neuregulin polypeptide.

Figure 1

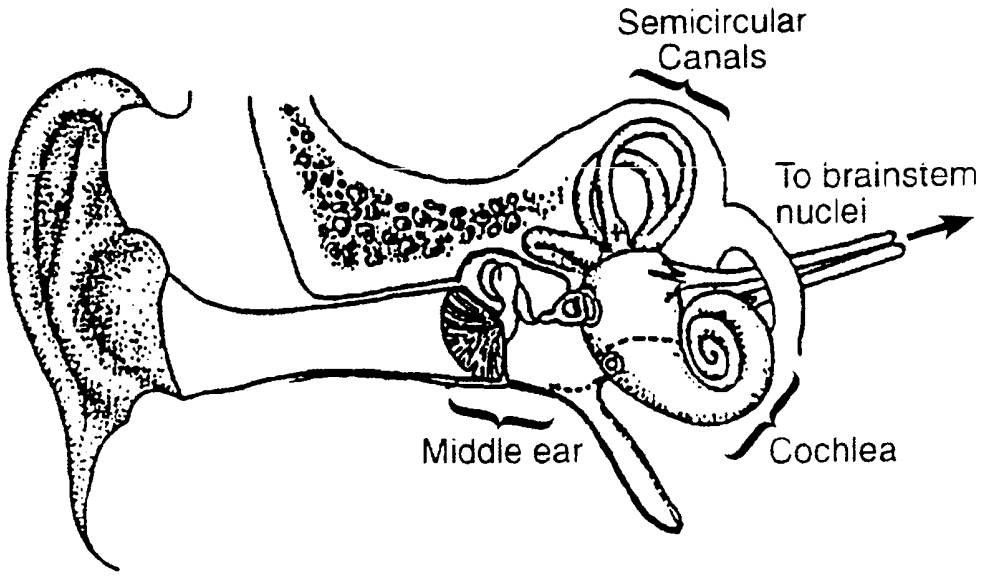

Semicircular
Canals
To brainstem
nuclei
Middle ear
Cochlea

Figure 2

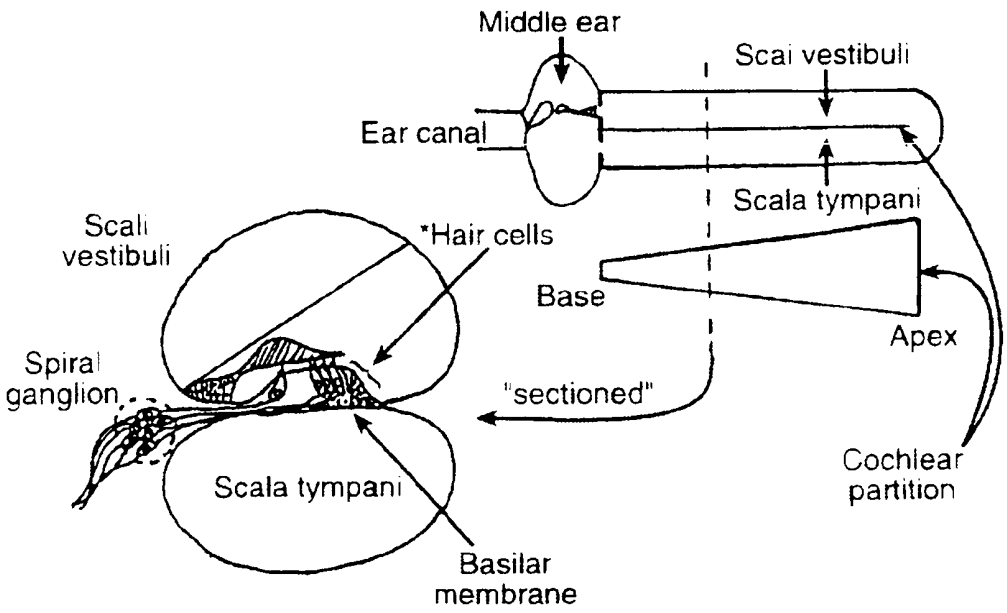

Middle ear
Scai vestibuli
Ear canal
Scala tympani
Scali vestibuli
*Hair cells
Base
Apex
Spiral ganglion
"sectioned"
Cochlear partition
Scala tympani
Basilar membrane

Figure 3

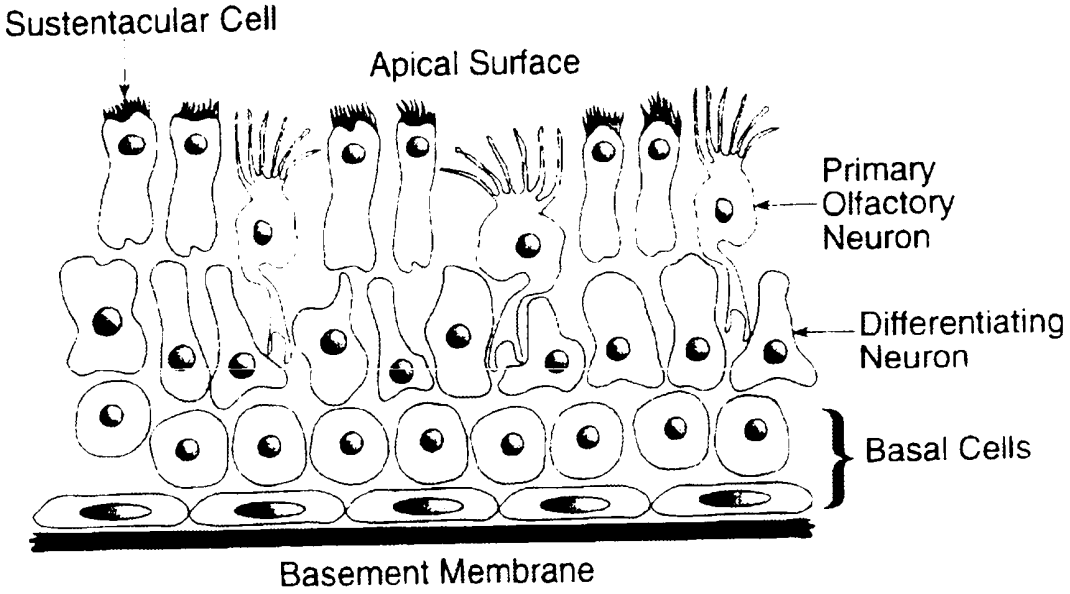


Figure 4

[rhGGF2, ng/ml]	% labelled cells
0	12.1
25	23.0
50	17.2
100	21.7

Figure 5

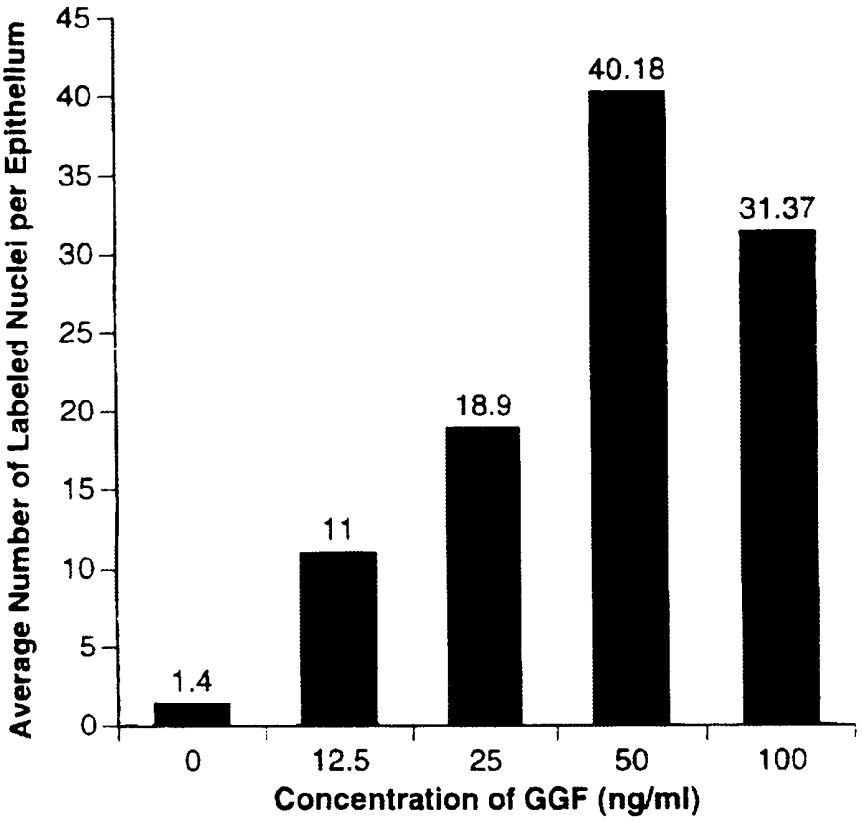

Average Number of Labeled Nuclei per Epithelium
45
40
35
30
25
20
15
10
5
0
1.4
11
18.9
40.18
31.37
0
12.5
25
50
100
Concentration of GGF (ng/ml)

Figure 6

Figure 7

Figure 8

Fig. 8A

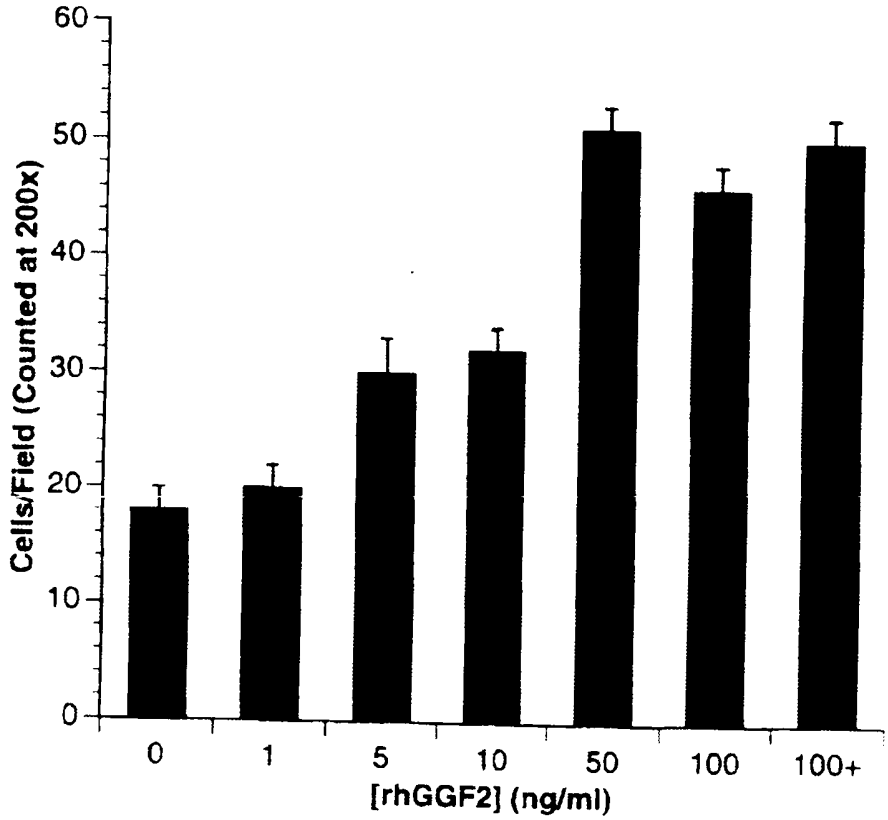


Fig. 8B

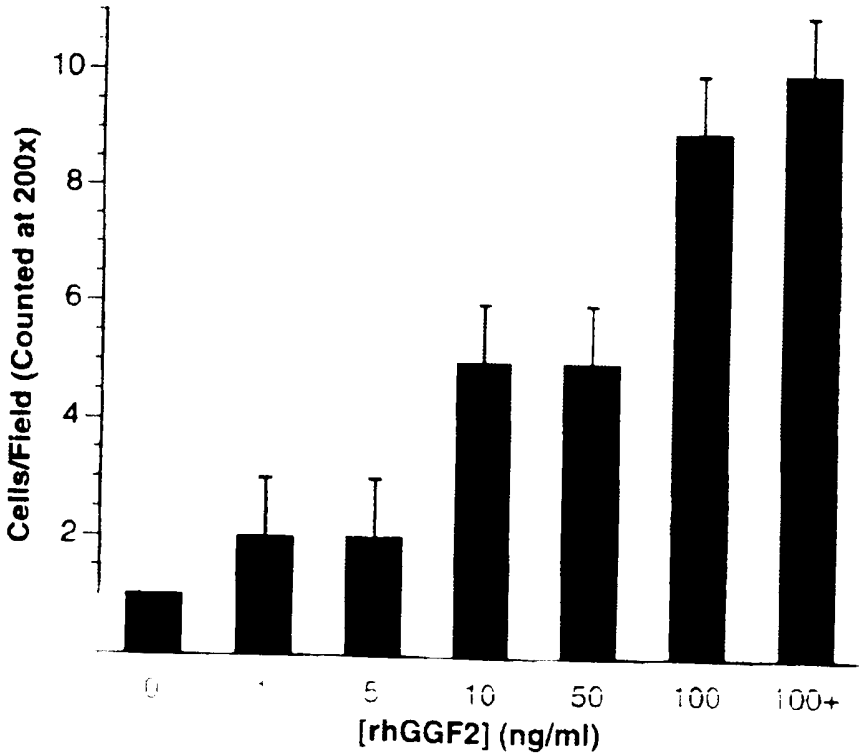
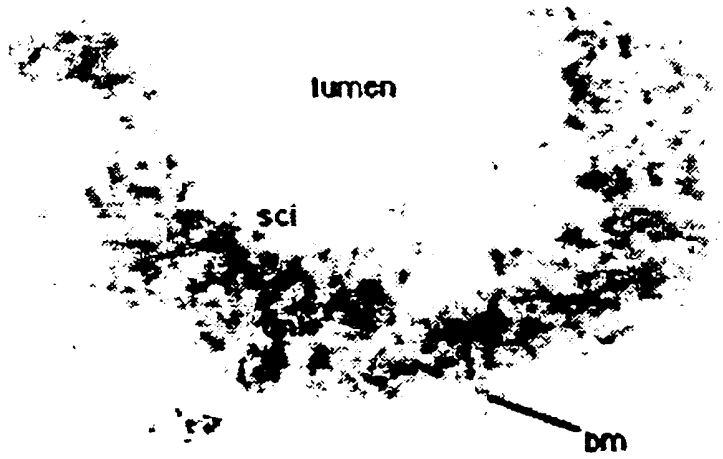


Figure 9



INTERNATIONAL SEARCH REPORT

International application No
PCT/US96/18031

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y, A	MARCHIONNI, M.A. Neu tack on neuregulin. Nature, 23 November 1995, Vol. 378, pages 334-335, see entire document.	1-28
Y	MAHANTHAPPA, N.K., ET AL. Peptide Growth Factor Control of Olfactory Neurogenesis and Neuron Survival In Vitro: Roles of EGF and TGF-βs. Neuron, February 1993, Vol. 10, pages 293-305, see entire document.	1-28
Y	DAVIS, J.G. ET AL. Use of the Teleost Saccule to Identify Genes Involved in Inner Ear Function. DNA and Cell Biology, 1995, Vol. 14, No. 10, pages 833-839, see entire document.	4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

[illegible] published [illegible] priority date claimed

[illegible] actual completion [illegible] international search

[illegible] mailing [illegible] international search report

28 JANUARY 1997

27 FEB 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer
Jacqueline Krikorian

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18031

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	GU, R. ET AL. Glial Growth Factor Enhances Supporting Cell Proliferation in Rodent Vestibular Epithelia Cultured in Isolation. Society for Neuroscience Abstracts, 1996, Vol. 22, p. 520, Abstract No. 213.2, see entire abstract.	1-28
Y	MAHANTHAPPA ET AL. The Role of Neuregulins in Olfactory Neurogenesis. Society for Neuroscience Abstracts, 1995, Vol. 221, p. 1512, Abstract No. 595.4, see entire abstract.	1-28
Y	YAMASHITA ET AL. Induction of Cell Proliferation in Mammalian Inner-ear Sensory Epithelia by Transforming Growth Factor α and Epidermal Growth Factor. Proc. Natl. Acad. Sci. (USA), 1995, Vol. 92, No. 8, pages 3152-3155, see entire document.	1-28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/18031

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US96/18031

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/00, 38/18, 31/70

A. CLASSIFICATION OF SUBJECT MATTER:
US CL

514/2, 12, 44

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

514/2, 12, 44

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

CAPlus, Medline, EMBASE, BIOSIS, WPIDS, APS
Search terms: sensory epithelial, erb b receptor, glial growth factor, neuregulin, hearing, ear, auditory, vestibular, ototoxic, Meissner, Paciminan

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-3, and 5-28, drawn to a method of treating non-visual sensory epithelial cells comprising using a polypeptide which binds to an erb B receptor.

Group II, claim 4, drawn to a method of treating non-visual sensory epithelial cells comprising using a DNA sequence encoding a polypeptide which binds to an erb B receptor.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The apparent special technical feature of the invention of Group I, claims 1-3 and 5-28, is treatment of non-visual sensory neurons by administering a polypeptide which binds to an erb B receptor. The apparent special technical feature of Group II, claim 4, is the use of a DNA coding sequence to treat non-visual sensory epithelial cells. The steps involved in the methods of Groups I and II are disparate and are reflective of the fundamental differences between proteins and nucleic acids. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Note that PCT Rule 13 does not provide for multiple methods within a single application.